Inhibiting Alternative Pathway Complement Activation by Targeting the Exosite of Factor D

Supplementary material

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SUPPLEMENTAL METHODS

Enzyme assay - FD hydrolysis of thioester benzyl substrate Z-Lys-SBzl was measured using DTNB (Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid)) in assay buffer (50 mM hepes pH 7.5 and 220 mM NaCl). Assay volume was 200 µl in assay buffer with final concentrations of 2 mM DTNB, 800 nM Z-Lys-SBzl, 80 nM FD, 800 nM antibodies or 20 mM DIFP. Hydrolysis rates were measured in a Spectramax Plus 384 spectrophotometer at 405 nm for 1.5 hours, readings taken every 30 seconds and plotted as time versus product using SoftMax Pro v5.2 software.

Biacore analysis of FD binding to AFD – Surface plasmon resonance (SPR) measurements of FD binding to AFD were performed on a Biacore® 4000 instrument (GE Healthcare) using a capture method to immobilize AFD. An anti-human Fab antibody (Human Fab Capture Kit 28-9583-25; GE Healthcare) was immobilized on a Series S CM5 chip following the protocol supplied by the manufacturer. Following capture of ~ 100 Resonance Units (RU) of AFD, sensorgrams were recorded for injection of solutions of human or cyno FD varied in concentration from 0.048 nM to 12.5 nM in 2-fold increments. Injections of 60 µL were made at a flow rate of 30 µL/min and dissociation was monitored for 10 minutes prior to regeneration between binding cycles using 3 M $MgCl_2$. Binding buffer contained 10 mM HEPES pH 7.2, 150 mM NaCl, 3 mM EDTA, and 0.01% Polysorbate 20 and the temperature was 25 °C. Sensorgrams (Supplemental Fig. S5A) were double-referenced by subtracting both the binding signal observed for FD on the capture antibody alone and for injection of buffer alone. Kinetic constants (Supplemental Fig. S5B) were calculated from the observed sensorgrams using Biacore4000 Evaluation Software v1.0. Binding of both human and cyno FD had on-rates at the detection limits of the instrument, and the K_D calculated for human FD is less than the practical limit (10 pM) for affinity measurement by biacore. The high affinities measured by SPR do not appear to reflect a contribution of the dextran matrix of the CM5 chip to binding since inclusion of 5 mg/mL carboxymethyl detran in the binding buffer did not change the calculated affinities. Consistent with the number of AFD Asp residues observed in the contact region with FD, the binding affinity was reduced by 4 orders of magnitude for SPR measurements at pH 5 (data not shown).

SUPPLEMENTAL TABLES

Table S1. Data collection and refinement statistics

	Human	Cynomolgus
Resolution range (Å)[a]	25 - 2.42	73.27 - 2.28
	(2.51 - 2.42)	(2.4 - 2.28)
Space group	P 43, 21, 2	C 1, 2, 1
Unit cell	132, 132, 180, 90, 90, 90	182, 80.8, 143, 90, 107, 90
Total reflections	242985	337138
Unique reflections	60502	90191
Multiplicity	4.0 (3.9)	3.7 (3.7)
Completeness (%)	98.60 (99.50)	99.8 (99.9)
I/sigma(I)	13.97 (2.3)	17.3 (2.8)
Wilson B-factor	46.2	37.3
R-sym[b]	0.099 (0.505)	0.047 (0.40)
R-factor/R-free[c]	0.207/0.266	0.196/0.239
No. reflections (F>0σ(F))	60430	90153
Number of atoms	10324	10837
Protein residues	1317	1310
Water molecules	378	845
RMS(bonds)	0.013	0.013
RMS(angles)	1.91	1.82
Ramachandran favored (%)	95	97
Ramachandran outliers (%)	0.56	0.40
Avg. B-factor protein/solvent	51.9/48.5	49.0/53.7

[a] Numbers in parentheses refer to the highest resolution shell.

[b] $R_{sym} = \Sigma|I-\langle I\rangle| / \Sigma I$. $\langle I\rangle$ is the average intensity of symmetry related observations of a unique reflection.

[c] $R = \Sigma|F_O-F_C| / \Sigma F_O$. R_{free} is calculated as R, but for 5 % of the reflections excluded from all refinement.

Table S2. Buried surfaces (Å^2) for the individual chains in the AFD : FD complex (human FD)

Chain L (LC)	Area (Å^2)	Chain H (HC)	Area (Å^2)	Chain A (FD)	Area (Å^2)
Asp 30	16.5	Thr 28	24.2	Asp 129A	29.9
Asp 32	36.1	Thr 30	10.1	Val 130	1.8
Asn 34	13.2	Asn 31	95.2	Ala 131	0.6
Tyr 36	7.0	Tyr 32	36.5	Pro 132	35.7
Leu 46	5.8	Gly 33	5.9	Asp 164	45.1
Ser 49	5.1	Trp 50	34.1	Arg 165	33.3
Arg 55	26.1	Asn 52	31.2	Ala 166	74.3
Leu 89	3.1	Thr 53	0.6	Thr 167	16.9
Ser 91	26.8	Tyr 54	107.1	Asn 169	42.4
Asp 92	51.7	Thr 55	26.0	Arg 170	97.0
Ser 93	16.7	Glu 57	5.5	Arg 170A	188.6
Leu 94	26.9	Glu 97	1.2	Thr 170B	57.8
Tyr 96	27.9	Glu 99	47.4	Asp 173	60.5
		Gly 100	25.8	Gly 174	33.3
		Gly 101	28.7	Ile 176	5.5
		Asn 109	5.3	Thr 177	3.3
				Glu 178	48.0
				Asn 222	2.3
				Arg 223	41.7
				Lys 223A	80.0
				Lys 224	2.8
Buried surface of chain L:	262.9 Å^2	Buried surface of chain H:	484.8 Å^2	Buried surface of chain A:	900.8 Å^2

SUPPLEMENTAL FIGURE LEGENDS

FIGURE S1. Effect of AFD on esterolytic activity of FD. Steady state kinetics of cleavage of a small esterolytic substrate, Z-Lys SBzl, by FD in the absence or presence of AFD, 8E2, control Fab or diisopropyl fluorophosphate (DIFP).

FIGURE S2. FD does not bind directly to C3b. SPR analysis, in which a fixed concentration of FD_{S195A} was injected over immobilized C3b in the presence or absence of FB. R.U. =resonance units.

FIGURE S3. Structures of AFD in complex with human and cynomolgus FD. *A*, structure of two human FD:AFD complexes in the asymmetric unit are shown as ribbon diagrams. Human FD is in white, AFD LC and HC are colored yellow and orange, respectively. *B*, Overlay of the two human FD:AFD complexes. *C*, structure of two cynomolgus FD:AFD complexes in the asymmetric unit. Cynomolgus FD is colored green, color code of AFD is similar to A.

FIGURE S4. Residues interacting at the FD - AFD interface are well represented by the electron density. The 2Fo-Fc electron density map of human FD in complex with AFD is contoured at 1s, coloring is according to Supplemental Fig. 3A.

FIGURE S5. Biacore analysis of binding affinity of human and cynomolgus FD to AFD. *A*, Biacore sensorgrams of human FD binding to AFD at neutral pH (pH 7.2). AFD was captured on a sensor chip derivatized with anti-human Fab antibody and Human FD was flowed over the chip in a concentration series of 0.048 nM - 12.5 nM. Colored lines are experimental data and black lines are calculated from analysis of the data according to a 1:1 binding model using Biacore4000 evaluation software. *B*, Binding constants calculated for human and cyno FD binding to AFD.

FIGURE S6. Sequence differences between human and cynomolgus factor D explain the cross-reactivity of AFD. *A*, structure of the human FD : AFD complex with residues that are different between human and cynomolgus FD shown as sticks and colored cyan (human) or magenta (cynomolgus). *B*, sequence alignment of human chymotrypsin, human FD and cynomolgus FD. Residue differences between human and cynomolgus FD are numbered and boxed. Chymotrypsin numbering is used.

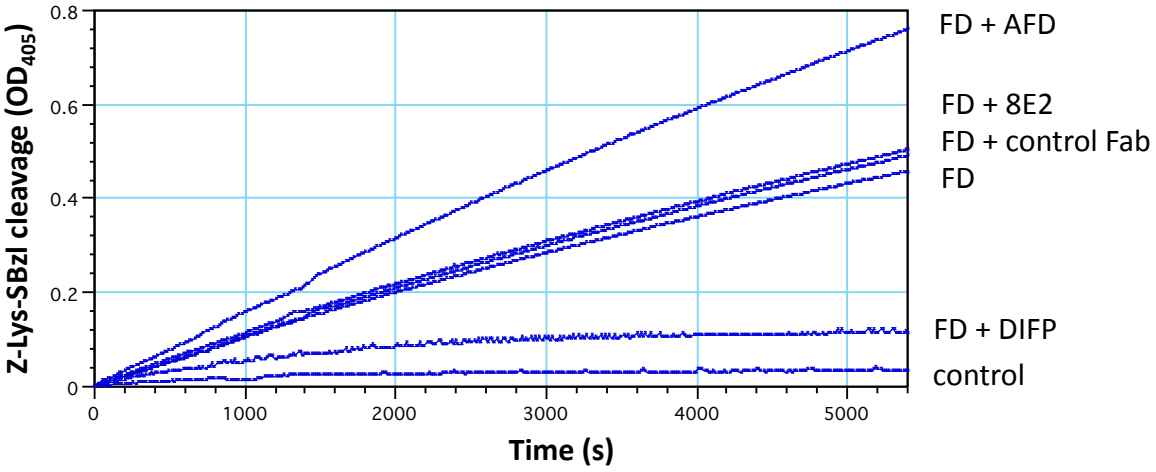

Z-Lys-SBzl cleavage (OD405)
0.8
0.6
0.4
0.2
0
0
1000
2000
3000
4000
5000
Time (s)
FD + AFD
FD + 8E2
FD + control Fab
FD
FD + DIFP
control

Fig. S1

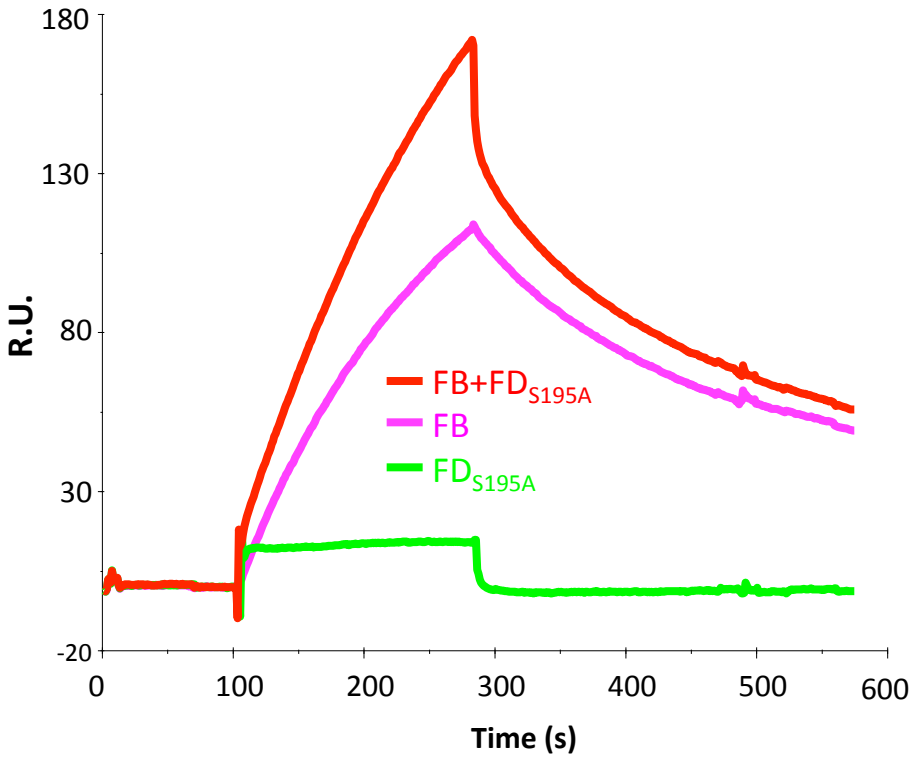

180
130
80
30
-20
R.U.
0
100
200
300
400
500
600
Time (s)
FB+FD$_{S195A}$
FB
FD$_{S195A}$

Fig. S2

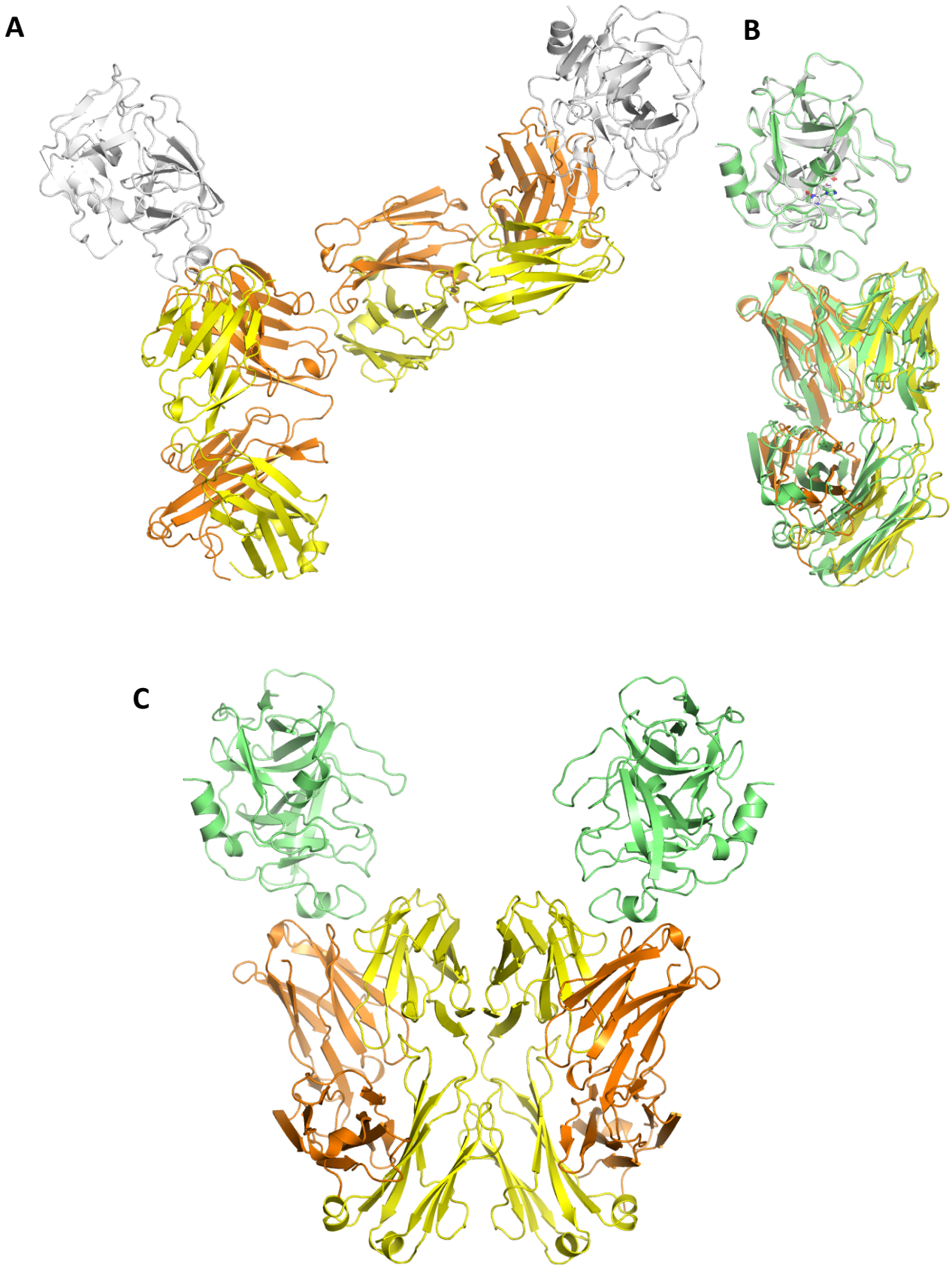


Fig. S3

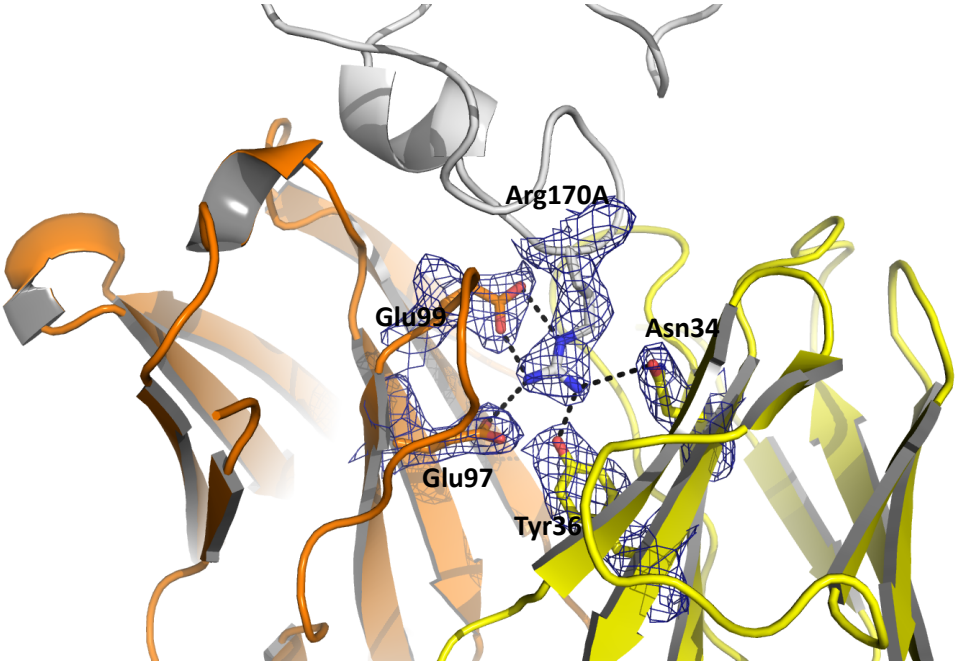
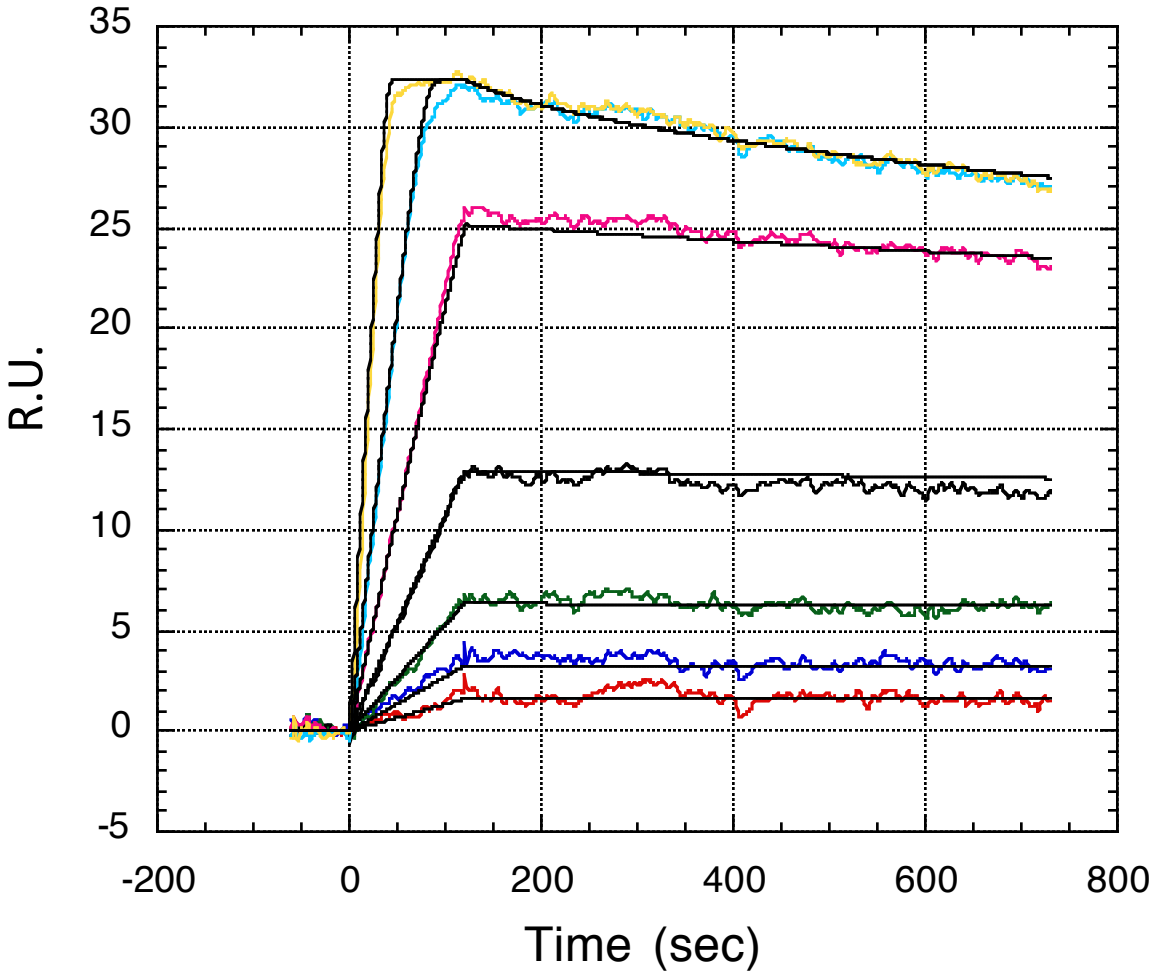


Fig. S4

A

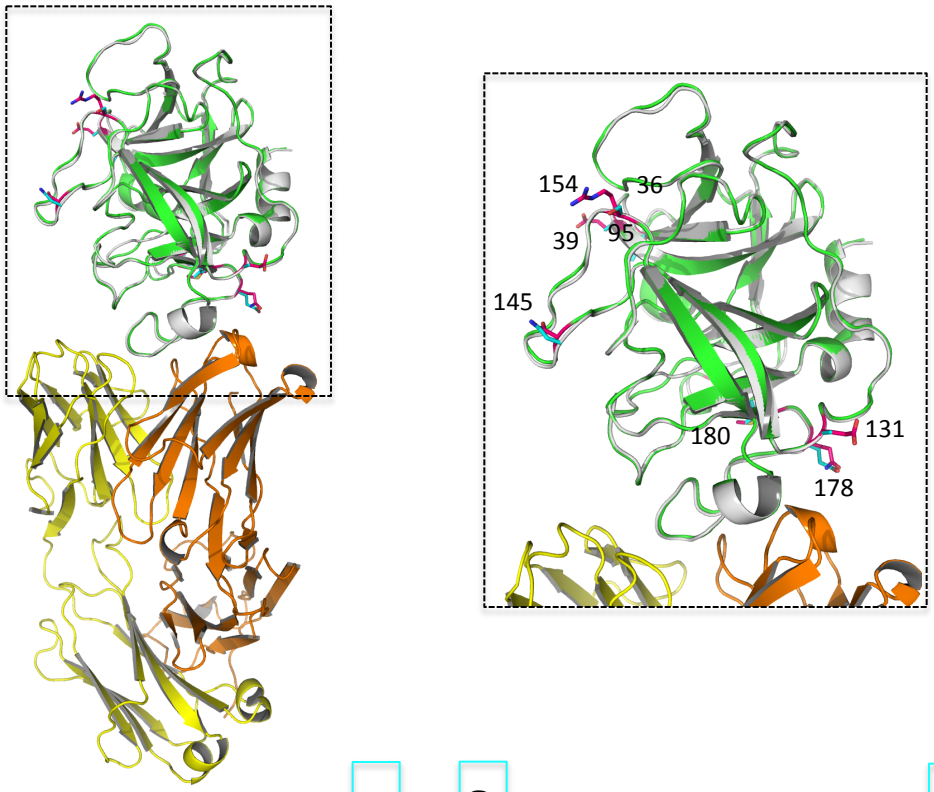


B

FD	k_a (1/M s)	k_d (1/s)	K_D (pM)	Chi^2
human	8.2e^{7}	3.3e^{-4}	4.0	0.6
cynomolgus	2.8e^{8}	8.5e^{-3}	30	0.5

Fig. S5

A


154
36
39
95
145
180
131
178

B

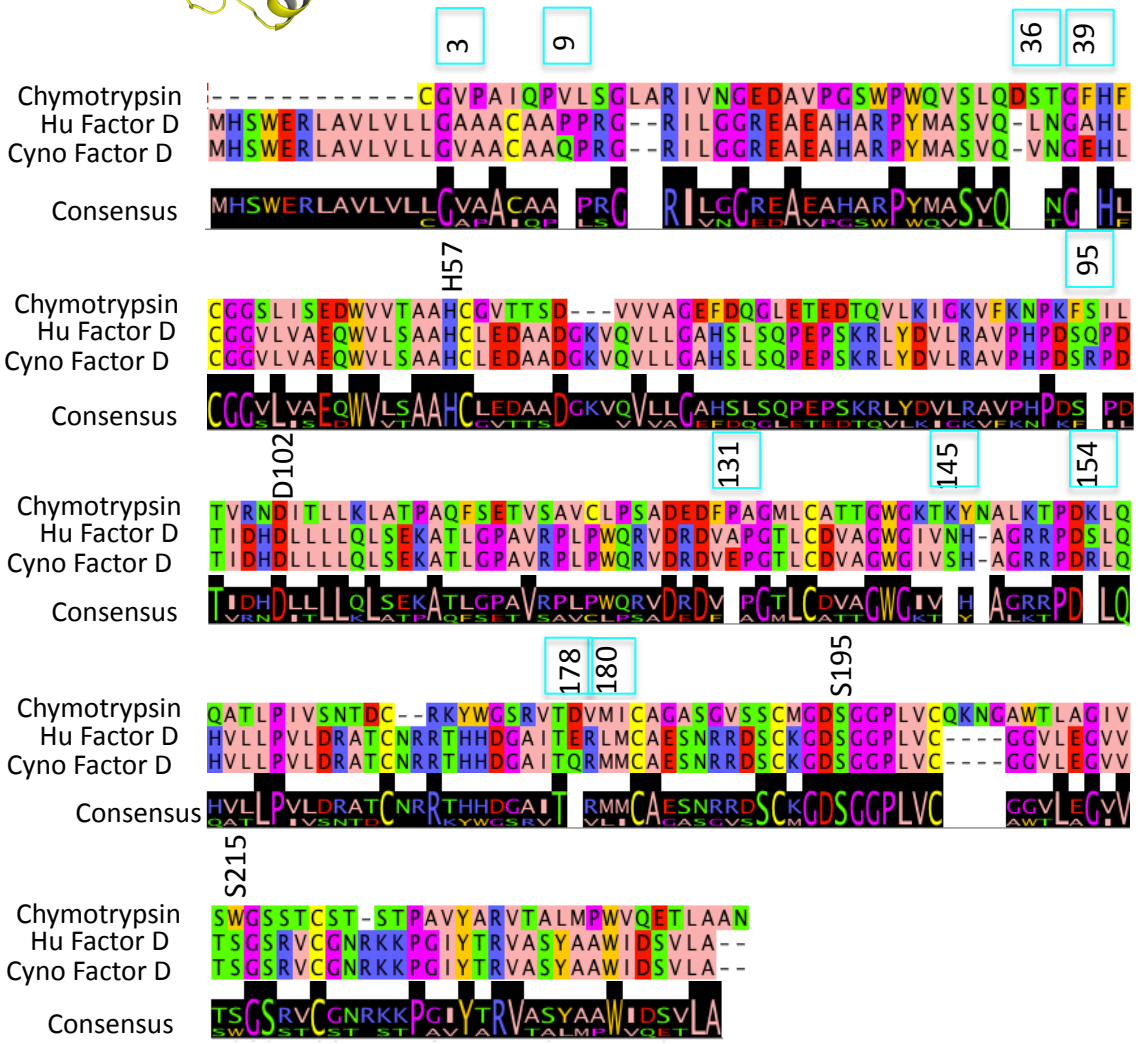

3
9
36
39
H57
95
D102
131
145
154
178
180
S195
S215
Chymotrypsin
Hu Factor D
Cyno Factor D
Consensus

Fig. S6